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Acrosome reaction inactivation in sea urchin sperm

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Abstract

Acrosome reaction inactivation (ARI) is a process that renders sperm irreversibly refractory to the egg jelly (the natural inducer of the acrosome reaction, AR). This process triggered by the egg jelly, is associated with an increase in $[Ca^{2+}]_i$. However, we show here that a rise in $[Ca^{2+}]_i$ alone is not sufficient to induce ARI, since artificially increasing $[Ca^{2+}]_i$ with either an ionophore or rising external pH, does not trigger ARI. Contrary to the AR which strictly requires Ca^{2+}, ARI can be triggered almost equally well by Sr^{2+}. On the other hand, Mn^{2+} inhibits ARI and, as we showed earlier, does not affect AR. These observations indicate that the mechanisms involved in ARI differ from those leading to AR. In addition, we report here that high external pH (a non-physiological inducer of AR) triggers the AR in previously inactivated sperm by opening the same Ca^{2+} channels activated by the egg jelly. Considering that the opening of Ca^{2+} channels is one of the earliest responses triggered by the egg jelly and that ARI requires the egg jelly receptor to be activated, we have concluded that ARI involves the uncoupling between the egg jelly receptor and Ca^{2+} channels. Furthermore, intracellular pH (pH_i) determinations, in the presence or absence of ionomycin to substitute for the uncoupled Ca^{2+} channels, indicate that pH_i regulation is also impaired in inactivated sperm. In conclusion, ARI is a manifestation of the uncoupling of the egg jelly receptor from the different ion transport systems required for the acrosome reaction. © 1998 Elsevier Science B.V.

Keywords: Acrosome reaction inactivation; Calcium ion, intracellular; Sperm; (Sea urchin)

1. Introduction

Sperm must undergo the acrosome reaction (AR) in order to fertilize an egg. The external coat of the egg known as the ''egg jelly'', or a factor purified from it, are the natural inducers of the AR in sea urchin sperm. They induce an influx of Ca^{2+} and Na^+, and efflux of K^+ and H^+ [1,2], which produce an increase in $[Ca^{2+}]_i$ [3–5] and pH_i [5,6], concur-

Abbreviations: AR, acrosome reaction; ARI, acrosome reaction inactivation; $[Ca^{2+}]_i$, intracellular calcium concentration; pH_i, intracellular pH; pH_e, external pH; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF/AM, acetoxymethyl ester of BCECF; fura-2/AM, acetoxymethyl ester of fura-2; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; TEA, tetraethylammonium; DMSO, dimethyl sulfoxide; ASW, artificial sea water; 0CaASW, Artificial sea water without Ca^{2+}

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rently with a transient K^+-sensitive hyperpolarization [7] followed by a steady Ca^{2+} dependent depolarization [8,9], among other changes. Ca^{2+} and K^+ channel blockers (verapamil and dihydropyridines, and TEA^+, respectively) inhibit the AR suggesting a fundamental role for these channels in the induction of acrosome reaction [1,2].

Apparently the activation of the egg jelly receptor is also involved in triggering what we have named the acrosome reaction inactivation or ARI [4]. This process renders sperm unresponsive to the egg jelly in an irreversible form, and cannot be triggered by non-natural inducers of AR [10]. Furthermore, it has been shown that ARI is a species specific event [11]. Interestingly, certain monoclonal antibodies against the 210 kDa plasma membrane protein, that is considered the best candidate for being the egg jelly receptor (at least one of its subunits), block the AR by producing a transient increase in $[Ca^{2+}]_i$ without affecting pH_i [3].

We have studied the role of Ca^{2+} channels and pH_i in triggering ARI and whether inactivation of Ca^{2+} channels, opened by the egg jelly, could be the underlying mechanism of this process. The opening of Ca^{2+} channels is one of the earliest responses associated to the AR [3,5], here we report that high external pH activates the same Ca^{2+} channels as the egg factor in previously inactivated sperm. Surprisingly, pH_i determinations indicate that pH_i regulation is also impaired in inactivated sperm. These results altogether suggest that ARI is due to a generalized uncoupling between the egg jelly receptor and the different ion transport systems involved in the AR. Preliminary results have been reported elsewhere [12].

2. Materials and methods

Strongylocentrotus purpuratus sea urchins and their gametes were obtained and kept as previously reported [4,5]. All experiments were done with sperm suspended in artificial sea water (ASW). Unless otherwise indicated, the composition of ASW was (in mM): NaCl 486, KCl 10, $NaHCO_3$ 2.4, $MgCl_2$ 56, $CaCl_2$ 10, EDTA 0.1 and Hepes 10 at pH 8.0 adjusted with NaOH. ASW0Ca had the same composition than ASW except that $CaCl_2$ was omitted. Sperm from fresh semen (50 μl) diluted 1:10 in ASWL (ASW with 1 mM Ca^{2+} and pH 7.0) were loaded with Fura-2 or 2′,7′-bis-(2 carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) using 12 μM of fura-2/acetoxymethyl ester (AM) (DMSO 0.6% final) or 10 μM BCECF/AM (DMSO 1.0% final) overnight at 4°C, respectively. Sperm suspensions were washed by diluting 10 times with ASWL and centrifuging 6 min at $1000 \times g$. Cells resuspended in the initial volume were kept on ice until used.

Fura-2 fluorescence was recorded and calibrated as previously reported [4,5]. Fura-2 signals are expressed as the fraction of saturation by Ca^{2+} (f) which is related to $[Ca^{2+}]_i$ by the following equation: $[Ca^{2+}]_i = K_d(f/(1-f))$. At the usual fura-2 concentrations used inside cells, f is quasi-linearly related to the total amount of Ca^{2+} in the cytoplasm [13], therefore f is a better indicator of Ca^{2+} influx than $[Ca^{2+}]_i$. The latter can be calculated using a K_d of 700 nM for sea urchin sperm [3]. In order to obtain fura-2 fluorescence ratios (340/380 nm) with a single wavelength spectrofluorometer we repeated the experiments at the two excitation wavelengths and calculated the ratio. The Ca^{2+} insensitive wavelength for fura-2 inside sperm was found to be 357 nm [5]. The rate of Mn^{2+} influx was estimated from the time course of fura-2 fluorescence quenching at 357 nm after adding 2 μM of ionomycin to obtain the maximal level of quenching. This Mn^{2+} influx activated by the egg jelly or factor was perfectly fitted by a single exponential plus a constant; the latter was required because Mn^{2+} quenched more fura-2 with ionomycin than egg jelly, probably because a small fraction of fura-2 was compartmentalized.

Internal pH was measured using the fluorescent pH sensitive dye, BCECF as we previously reported [5]. External pH (pH_e) was increased from its normal value of 8 to 9.1–9.3 by adding 10 mM CHES (final concentration) at a pH of 10.5 and normal pH was recovered by adding 12.5 mM MES (final concentration) at a pH of 5.5. This approach was preferred to the addition of NaOH [14] because changes in pH_e were more stable and reproducible, and these pH buffers do not by themselves interfere with AR (see Section 3).

AR determination, as well as preparation and quantification of the egg jelly and factor were carried out as previously described [9]. The concentration of

the egg factor used was always maximal, as judged by the levels of AR, and never exceeded 1.5% of the recording volume. The percentages shown next to the fluorescence traces indicate AR scored at the indicated time. All statistics represent the mean ± standard error of the mean with the number of independent experiments indicated in parenthesis.

Fura-2/AM and BCECF/AM were purchased from Molecular Probes (Eugene, OR). CHES and MES were from Sigma and the rest of the chemicals were of the best grade commercially available.

3. Results

3.1. ARI does not involve an increase in pH_i

AR requires a concomitant increment of both $[Ca^{2+}]_i$ and pH_i. Here, we studied the role of pH_i in the acrosome reaction inactivation. It is evident that the egg factor required Ca^{2+} influx to rise pH_i since this did not occur until external Ca^{2+} was added (Fig. 1(B)). Addition of external Ca^{2+} by itself did not produce any effect on resting pH_i (Fig. 1(A)). Furthermore, when $[Ca^{2+}]_i$ was clamped to very low levels with a permeable Ca^{2+} chelator, the egg factor did not increase pH_i either (not shown). These results indicate that the rise in pH_i associated to the AR is completely Ca^{2+} dependent.

When external Ca^{2+} was set to 2 mM, a condition that triggers ARI upon exposure to the egg factor [4], no significant increase in pH_i was induced by the natural inducer of the AR, neither by restoring the normal external $[Ca^{2+}]$ (Fig. 1(C)). This suggests that an increase in pH_i, which is needed to achieve AR, is not required by the egg factor to trigger ARI [3,5].

ARI is associated with a transient increase in $[Ca^{2+}]_i$ [4], as opposed to the AR which involves a sustained increase in $[Ca^{2+}]_i$ [3,5]. The transient nature of such rise in $[Ca^{2+}]_i$ could be generated either by a reduction in Ca^{2+} influx or an enhancement of the rate of Ca^{2+} removal. $[Ca^{2+}]_i$ measurements alone could not differentiate between these two possibilities. In order to study Ca^{2+} influx during ARI we employed Mn^{2+}, a well known Ca^{2+} surrogate [5,15,16]. At rest, Mn^{2+} influx was extremely small and very slow (diamonds, Fig. 2), suggesting that sperm have a very small Ca^{2+} influx at rest. Mn^{2+}

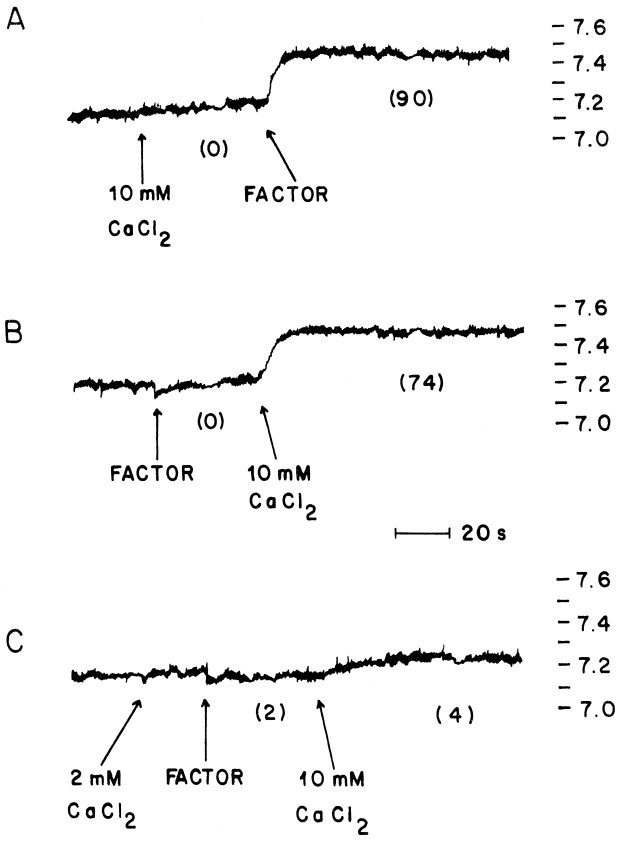

Fig. 1. Effect of acrosome reaction inactivation on pH_i. (A) Sea urchin sperm loaded with BCECF in 0CaASW with 0.5 mM of $CaCl_2$ were exposed to the egg factor at different external Ca^{2+} concentrations. (A) Normal external $[Ca^{2+}]$ was restored by adding 10 mM $CaCl_2$ where indicated before the addition of the egg factor. (B) Egg factor was added where indicated, and was followed by the addition of 10 mM $CaCl_2$ 30 s later. (C) To induce ARI, the egg factor was added but in the present of 2 mM external $[Ca^{2+}]$ followed by 10 mM $CaCl_2$. The numbers on the left side of the records are the pH_i values reported by BCECF. The numbers in parenthesis are the acrosome reaction attained where indicated. This is a representative experiment of 5.

influx increased several fold when sperm were exposed to the egg factor (squares, Fig. 2), in agreement with the opening of Ca^{2+} permeable channels induced by this egg component [5]. If the egg factor was added to sperm in sea water without Ca^{2+} and 30 s. later $[Ca^{2+}]_e$ was restored to its normal levels, Mn^{2+} influx and the AR were very similar to what was observed in normally reacted sperm (circles, Fig. 2). However, if the egg factor was added in the presence of 2 mM $[Ca^{2+}]_e$, a condition that induces ARI [4], Mn^{2+} uptake was smaller and at a lower rate

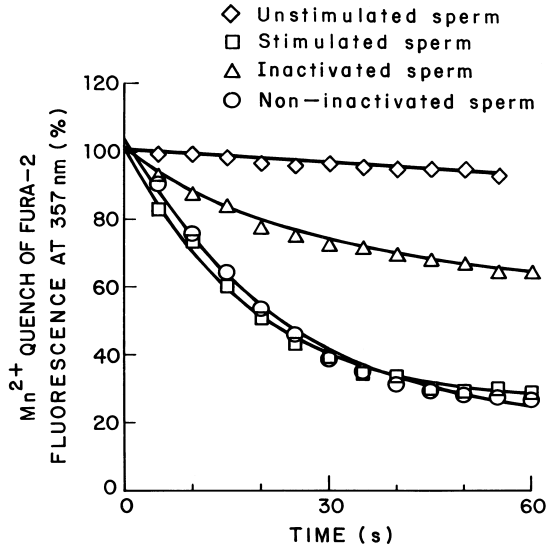

Fig. 2. ARI involves a reduced activity of Ca^{2+} channels. Sea urchin sperm loaded with fura-2 were excited at 357 nm (Ca^{2+}-insensitive wavelength) and the time course of quenching of fura-2 by adding 3 mM Mn^{2+} was recorded during 60 s for sperm under different conditions. Basal Mn^{2+} influx (diamonds) was obtained by adding Mn^{2+} to unstimulated sperm in normal ASW. Activation of Ca^{2+} channels by the egg factor in normal ASW was recorded by adding Mn^{2+} 30 s after the egg factor (squares). Activity of Ca^{2+} channels in non-inactivated sperm was determined by adding Mn^{2+} to sperm in 0CaASW that had been exposed to the egg factor for 60 s plus 10 mM $CaCl_2$ for another 30 s (circles). Mn^{2+} influx in inactivated sperm was obtained by adding Mn^{2+} after sperm in 0CaASW with 2 mM $CaCl_2$ were incubated with the egg factor for 60 s plus 10 mM $CaCl_2$ for another 30 s (triangles). The effect of the Mn^{2+} addition on the fura-2 fluorescence is shown at 5 s intervals (although fluorescence was sampled each 0.5 s). The time course of quenching was fitted to the following equation: $B + Aexp(-kt)$ where A represents the compartment sensitive to the egg factor and B the compartment accessible only after adding ionomycin. At the end of each record 3 μM ionomycin was added and the fluorescence obtained was considered as 100% quenching of fura-2 fluorescence (A + B).

(triangles, Fig. 2). These data (Table 1) indicate that there is an important inhibition of the Ca^{2+} influx in inactivated sperm suggesting that this could be the reason for the smaller change in $[Ca^{2+}]_i$ observed under this condition.

3.2. Inactivation of Ca^{2+} influx and of the AR occur as a function of time and $[Ca^{2+}]_e$

The effect of $[Ca^{2+}]_e$ on inactivation of Ca^{2+} influx and AR (Fig. 3) was studied by adding the egg factor to sperm suspended in 0CaASW, 0CaASW plus 0.5 mM Ca^{2+} or 0CaASW plus 1.0 mM Ca^{2+}, and determining the saturation of fura-2 (f) and the AR, 10 and 60 s after restoring $[Ca^{2+}]_e$ to normal, respectively. The egg factor was added at time zero and $[Ca^{2+}]_e$ was increased to its normal value of 10 mM $CaCl_2$ at the times indicated in Fig. 3. External Ca^{2+} hastened the rate of inactivation of both Ca^{2+} influx and the AR, the effect being more pronounced on the latter (Table 2). Both rates of inactivation for AR and Ca^{2+} influx were increased by elevating $[Ca^{2+}]_e$, but it was ARI that showed faster kinetics and higher sensitivity to $[Ca^{2+}]_e$ (Table 2). This suggests that ARI is not the consequence of the inactivation of the Ca^{2+} channels opened by the egg factor, in agreement with data that will be shown below.

3.3. Divalent cation selectivity of the AR inactivation

To further test the role of Ca^{2+} channels in the AR inactivation we studied if permeant divalent cations could substitute for Ca^{2+} in this process. Sea urchin sperm suspended in 0CaASW only, or in 0CaASW with 2 mM of either Ca^{2+}, Sr^{2+}, Ba^{2+} or Mn^{2+} were challenged with egg jelly, 10 mM $CaCl_2$ was added 1 min later and the AR scored 1 min after the addition of Ca^{2+} (Fig. 4(A)). The percentages of sperm showing AR ($n = 3$) were $72 \pm 2.0\%$ in ASW0Ca; $0.6 \pm 3.4\%$ in ASW0Ca with 2 mM of $CaCl_2$; $4.6 \pm 3.4\%$ with 2 mM $SrCl_2$; $51.3 \pm 23.7\%$ with 2 mM $BaCl_2$ and $98.6 \pm 0.9\%$ with 2 mM $MnCl_2$. Sperm of the

Table 1
Effect of acrosome reaction inactivation on Ca^{2+} influx indicated by Mn^{2+} uptake

	Mn^{2+} uptake (%)	Rate of Mn^{2+} uptake ($t_{1/2,S}$)
SPERM (10CaASW)	13.0 ± 0.1	64 ± 4.3
SPERM (10CaASW) + J	74.0 ± 3.6	15.5 ± 1.1
SPERM (0CaASW) + J + Ca^{2+}	77.3 ± 4.3	15.9 ± 0.0
SPERM (2CaASW) + J + Ca^{2+}	54.4 ± 7.7	27.7 ± 5.2

Sperm were incubated with egg jelly (where inidcated) for 30 s under different external $[Ca^{2+}]$ followed by the addition of Ca to restores the normal external $[Ca^{2+}]$ (10 mM). A minute later, 3 mM $MnCl_2$ was added to assess the state of Ca^{2+} channels. The time course of quenching of fura-2 fluorescence by Mn^{2+} was fitted as indicated in Section 2. Quenching in the presence of ionomycin was used as 100%, $n = 3$.

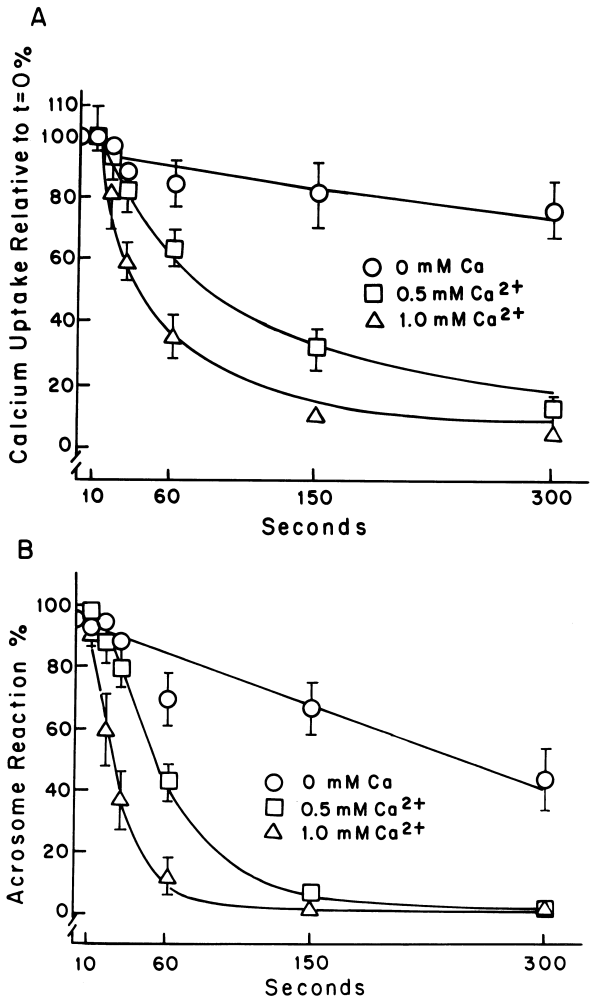

Fig. 3. External Ca^{2+} accelerates inactivation of sea urchin sperm. Sperm incubated in 0CaASW (circles) or either 0CaASW with 0.5 mM Ca^{2+} (squares) or 1.0 mM Ca^{2+} (triangles) with the egg factor for the indicated times. Thereafter, $CaCl_2$ was added to a final concentration of 10 mM to the sperm suspension, and the increase in the fraction of saturation of fura-2 measured 10 s later (A) or the AR 1 min later (B). SEM is not shown if smaller than the symbol, $n = 3$.

same batches had a mean AR of $96.6 \pm 1.4\%$ in normal ASW. These results indicate that the potency to induce AR inactivation was $Ca^{2+} \cong Sr^{2+} > Ba^{2+}$ and surprisingly, Mn^{2+} not only did not induced any inactivation but inhibited the inactivation induced by the egg factor in ASW0Ca. This sequence was not altered if the time between the egg jelly addition and 10 mM $CaCl_2$ was varied between 30 and 120 s (not shown). We tested if 10 mM of these cations could support the egg factor-induced AR and none of them did it except for Ca^{2+}. Furthermore, 2 mM of any of these cations did not inhibit the AR in normal external $[Ca^{2+}]$ (not shown). These data suggest that the egg factor triggers AR and the inactivation of the AR by two different mechanisms.

In the presence of 2 mM Mn^{2+} or Ca^{2+}, the addition of egg factor did not increase pH_i significantly. However, the former did not induce ARI as the latter did. As expected, the subsequent addition of 8 mM $CaCl_2$ produced the normal change in pH_i associated to the AR only in the case of 2 mM Mn^{2+} (not shown).

The inhibition of ARI by Mn^{2+} (Fig. 4(A)) clearly indicates that the Ca^{2+} dependency of this process does not reflect a requirement for Ca^{2+} for the binding of the egg factor to its receptor. The most plausible explanation arises after considering that Mn^{2+} is an open channel inhibitor of Ca^{2+} channels [15]. In the presence of 2 mM Mn^{2+}, Ca^{2+} influx will be strongly reduced, but when 10 mM Ca^{2+} is added, Mn^{2+} will be displaced from Ca^{2+} channels allowing enough Ca^{2+} influx for the AR to occur [5], this also implies that influx of divalent cations (Ca^{2+} or Sr^{2+}) is required for ARI. To test if increasing $[Ca^{2+}]_i$ alone was enough to trigger inactivation, $[Ca^{2+}]_i$ was elevated above the levels observed during ARI by adding 450 nM of ionomycin (Fig. 4(B)). At this concentration the ionophore produced a slow and small change in pH_i (not shown), and did not trigger any AR at this concentration, as expected from previous reports for *S. purpuratus* sperm [5]. The subsequent addition of the egg factor further increased $[Ca^{2+}]_i$ and normal levels of AR were attained ($n = 5$). It can be concluded that increasing $[Ca^{2+}]_i$ does not produce ARI unless it is associated to the activation of the receptor for the egg factor.

Table 2
Time in seconds to reach 50% inactivation of either acrosome reaction or Ca^{2+} influx. Experimental conditions as described in Fig. 3, $n = 3$

	Incativation of acrosome reaction (s)	Inactivation of Ca^{2+} influx (s)
0CaASW	294 ± 62	1197 ± 723
0CaASW + 0.5 mM Ca^{2+}	53.0 ± 6	89.0 ± 17
0CaASW + 1.0 mM Ca^{2+}	26.0 ± 4	38.0 ± 7

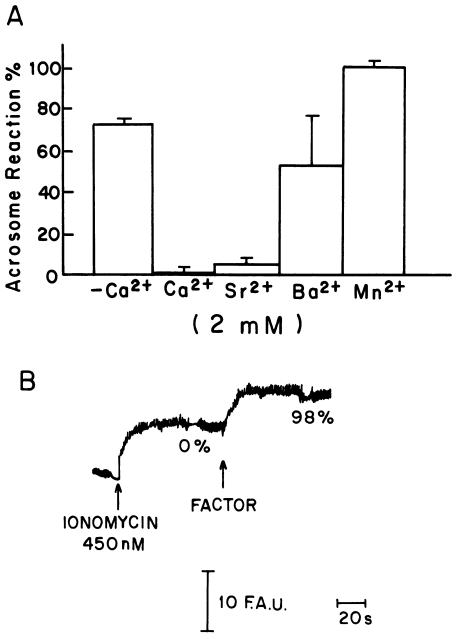

Fig. 4. An increase in $[Ca^{2+}]_i$ is not necessary nor sufficient to cause AR inactivation in sea urchin sperm. (A) The specificity for Ca^{2+} of AR inactivation was tested by incubating sperm with the egg factor in 0CaASW with 2 mM of either $CaCl_2$, $SrCl_2$, $BaCl_2$ or $MnCl_2$ for 1 min followed by addition of 10 mM $CaCl_2$. AR was scored 1 min after the addition of $CaCl_2$. The results are compared with respect to the inactivation obtained in the absence of $CaCl_2$ added during the incubation with the egg factor ($-Ca^{2+}$). Mean ± SEM, $n = 3$. (B) Effect of 450 nM ionomycin on $[Ca^{2+}]_i$ in sea urchin sperm loaded with fura-2 followed by the addition of the egg factor 1 min later. The percentage of reacted sperm are indicated. F.A.U. = fluorescence arbitrary units. Time scale in seconds is indicated, $n = 5$.

3.4. Effect of high pH_e on $[Ca^{2+}]_i$ and pH_i of sea urchin

It seemed then, that ARI requires activation of the egg jelly receptor. To test this we checked if elevated pH_e, a condition that triggers AR and does not involve the egg jelly receptor, could be affected by ARI. External pH was increased from 8.0 (its normal value) to 9.1–9.3, to induce AR [14,17,18]. This was done as indicated in Section 2 by adding CHES (10 mM, pH 10.5), and normal pH_e was recovered by adding MES (12.5 mM, pH 5.5). Fig. 5(A) shows that these pH buffers did not interfere with the egg factor induced AR provided that pH_e was returned to its normal value. Fig. 5 also compares the effect of high pH_e and the egg factor on $[Ca^{2+}]_i$ (B) and pH_i (C), respectively. The fura-2 fluorescence ratio at 340/380 nm excitation wavelengths showed that the changes in $[Ca^{2+}]_i$ produced by high pH_e and the egg factor were different. High pH_e always produced a biphasic change in $[Ca^{2+}]_i$, an initial fast and small increase in $[Ca^{2+}]_i$ followed, after a variable time of a few seconds, by a much slower and larger increase which virtually saturated fura-2 (Fig. 5(B)). Both, an elevated pH_e and the egg factor increased pH_i to the same extent, however the egg factor induced a faster

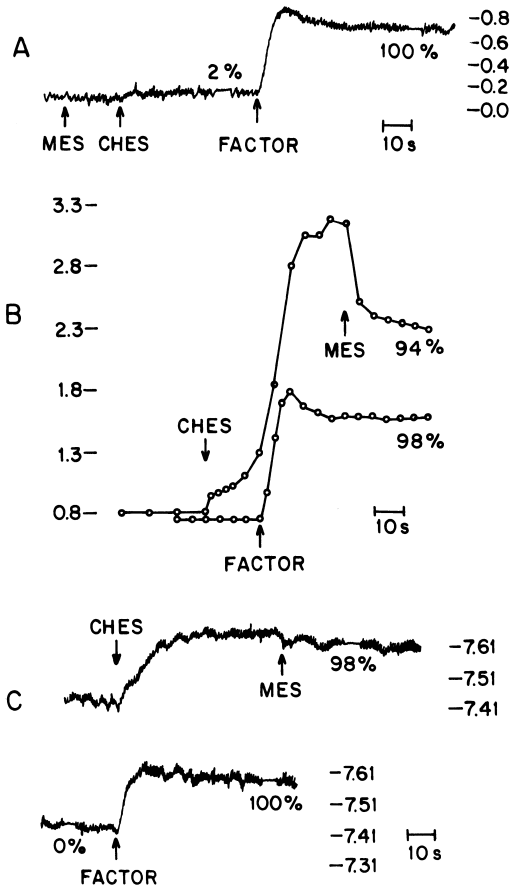

Fig. 5. High external pH increases $[Ca^{2+}]_i$ and pH_i. (A) External pH was reduced by adding 12.5 mM MES from a 1 M stock solution (pH 5.5) for the time indicated, this was followed by 10 mM CHES from a 1 M stock solution (pH = 10.5) to restore normal external pH. The fraction of saturation of fura-2, f, is indicated on the right side of the record. (B) Raw fura-2 340/380 nm ratios comparing the effect of the egg factor and elevated pH_e (9.2) using CHES. Similar AR were obtained despite different levels of $[Ca^{2+}]_i$. (C) Changes of pH_i induced by egg factor or high pH_e (CHES) on sperm loaded with BCECF. Time scale indicated in seconds, $n = 5$ for all experiments.

rise in pH_i (Fig. 5(C)). Increasing external K^+ to 30–40 mM or adding 10 μM nisoldipine, conditions that completely inhibit the egg factor induced AR [5,10] did not block the AR induced by high pH_e (not shown). Nevertheless, these inhibitory conditions did increase the time required to see the second rise in $[Ca^{2+}]_i$ associated with high pH_e (not shown, see discussion). These results imply that high pH_e induces the AR by a mechanism that does not involve activation of the egg jelly receptor, as the egg factor does.

3.5. High pH_e activates the same Ca^{2+} channels involved in the egg factor induced AR

Apparently, AR induced by high pH_e does not involve activation of the egg jelly receptor, however, it was not clear if Ca^{2+} influx occurred through different or the same Ca^{2+} channels activated by the egg factor. We have previously shown that Mn^{2+} can be used to distinguish two different Ca^{2+} channels activated by the egg factor [5]. Fig. 6 shows that both Ca^{2+} and Mn^{2+} influx were stimulated by high pH_e (Fig. 6(A)) in a very similar way as what has been shown for the egg factor (Fig. 6(B)). This indicates that the egg factor and elevated pH_e activate the same Ca^{2+} channels despite the egg jelly receptor not being involved in high pH_e-induced AR.

3.6. High pH_e overcomes the refractory state triggered by the egg factor

In agreement with ARI requiring an activated egg jelly receptor, high pH_e did not inactivate sperm in 2 mM external $[Ca^{2+}]_e$, in spite of the fact it increased $[Ca^{2+}]_i$ (Fig. 7(A)). Considering that high pH_e triggered the AR by activating the same Ca^{2+} channels as the egg jelly (Fig. 6), we tested if ARI originates from an irreversible inactivation of Ca^{2+} channels by increasing pH_e in previously inactivated sperm. Fig. 7(B) shows that high pH_e not only triggered the normal levels of AR in previously inactivated sperm but interestingly, its associated increase in $[Ca^{2+}]_i$ was much faster than observed in control sperm (compare with Fig. 5(B)). To test whether Ca^{2+} channel inactivation is the limiting step, a Ca^{2+} ionophore was used to bypass Ca^{2+} influx. Fig. 7(C) shows that ionomycin (3 μM) could not initially trigger AR in previously inactivated sperm even though it raised $[Ca^{2+}]_i$ to the levels associated with the egg jelly-induced AR. When this experiment was repeated but measuring pH_i, it became clear that ionomycin triggers AR but at a much slower rate, reflecting a very slow increase in pH_i (Fig. 8(A)). Unexpectedly, the ability of ionomycin to increase pH_i in inactivated sperm (Fig. 8(B)) is slower compared with control sperm. Nevertheless, natural inducers of AR rise pH_i much faster than ionomycin (Fig. 5(C)). This suggests that ARI also impairs the rise in pH_i. However, the addition to previously inactivated sperm of 10 mM NH_4Cl, to increase pH_i [5], together with 8 mM $CaCl_2$ (to restore normal external $[Ca^{2+}]$), did not clearly overcome the refractory state (not shown).

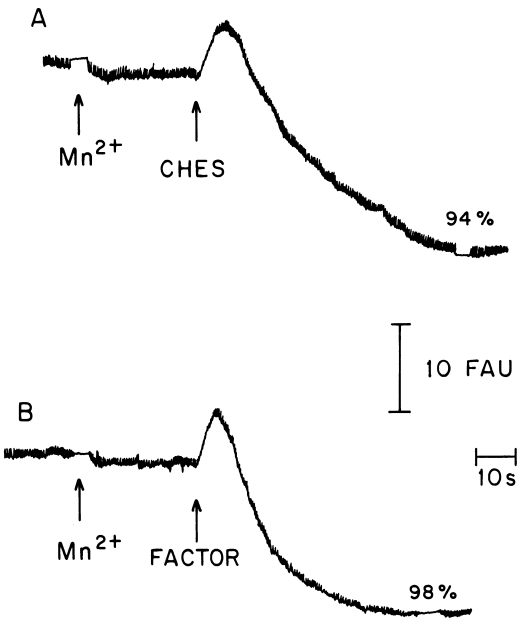

Fig. 6. High external pH increases Mn^{2+} influx. (A) Stimulation of Mn^{2+} influx by pH_e was tested by adding 1 mM Mn^{2+} followed by 10 mM CHES (pH 10.5) where indicated. Normal pH_e was restored 30 or 40 s later by adding 12.5 mM MES (not shown), $n = 6$. B) Mn^{2+} influx stimulated by the egg factor was obtained as previously reported [5] using 3 mM Mn^{2+}. Fluorescence arbitrary units are shown (FAU) since no calibration was attempted in the presence of Mn^{2+}.

To test the role of cAMP in ARI, we took advantage of Sr^{2+} being almost as potent as Ca^{2+} in triggering ARI (Sr^{2+} cannot substitute for Ca^{2+} in AR), to check if the egg factor was able to increase cAMP supported by Sr^{2+}. The result was negative, the cAMP increase showed a strict requirement for

external Ca^{2+} (not shown), suggesting that this nucleotide is not involved in triggering ARI in sea urchin sperm.

These observations taken altogether suggest that ARI does not involve inactivation of Ca^{2+} channels,

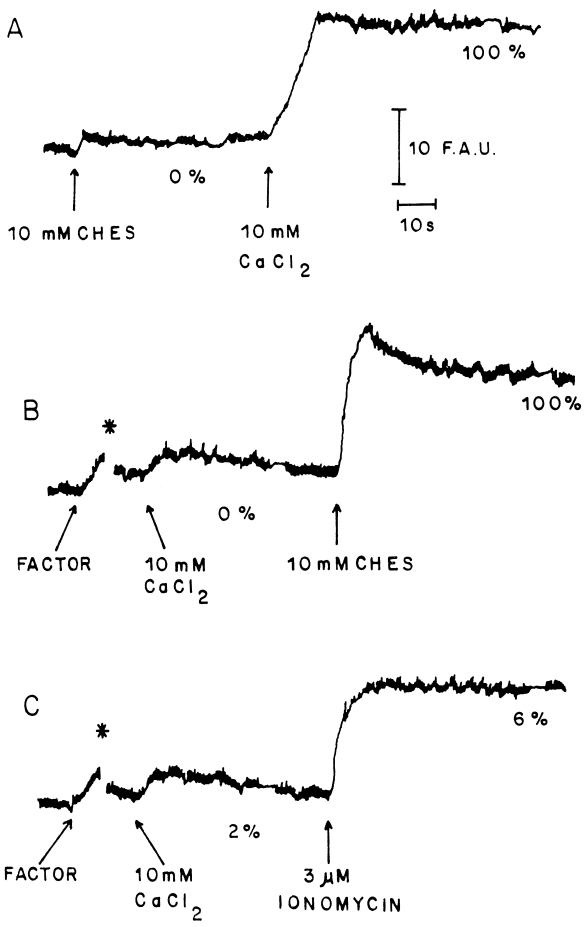

Fig. 7. Effect of high external pH on previously inactivated sperm. (A) Elevated external pH does not produce ARI. Fura-2 fluorescence excited at 340 nm of sperm in 0CaASW with 2 mM $CaCl_2$ were exposed to high pH_e for 50 s followed by $CaCl_2$ addition to 10 mM final, where indicated. Normal acrosome reaction was attained as shown. (B) Elevating pH_e triggers acrosome reaction in previously inactivated sperm. Egg factor was added to sperm in 0CaASW with 2 mM $CaCl_2$ and recording was halted for 40 s (∗) to allow inactivation of sperm, indicated by the absence of acrosome reaction after restoring normal $[Ca^{2+}]_e$, pH_e was increased to 9.15 by adding 10 mM CHES (pH 10.5) where indicated. High pH_e increased $[Ca^{2+}]_i$ without the characteristic lag found in native sperm. (C) same condition as in (B) except that ionomycin was used here to increase $[Ca^{2+}]_i$. No significant AR was elicited under this condition. Time scale in seconds applies to all records. Percentages of AR attained are indicated.

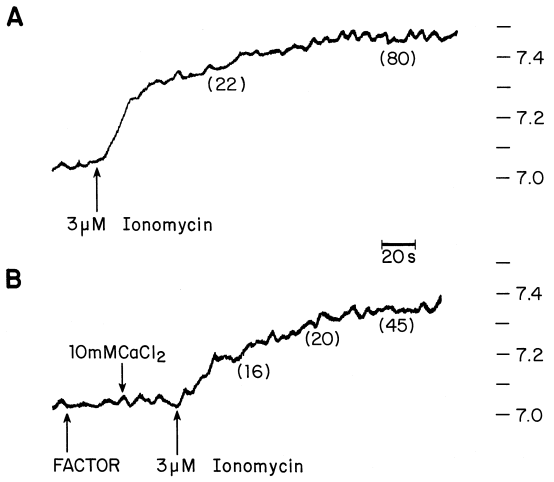

Fig. 8. Ionomycin induced pH_i changes in control (a) and previously inactivated sperm (B). (A) Ionomycin (3 µM) was used to increase $[Ca^{2+}]_i$ in control sperm suspended in ASW as shown in Fig. 7(C), except that pH_i was measured here instead. It became evident, when prolonged recordings were made, that ionomycin can trigger AR provided it increases pH_i as well as Ca^{2+}. However, pH_i elevates slower than $[Ca^{2+}]_i$ therefore, the former becomes the limiting step for the ionomycin-induced AR. Ionomycin increases pH_i faster in control sperm (A) than in sperm inactivated in 2 mM $CaCl_2$ after adding Factor (B). Accordingly, AR was attained sooner in control than in inactivated sperm ($n = 5$).

and indicate that artificially increasing Ca^{2+} influx or pH_i separately is not enough to overcome the refractory state induced by the egg factor. However, normal AR is evident when pH_i and $[Ca^{2+}]_i$ are risen concomitantly in inactivated sperm. This implies that the exocytosis machinery is not affected by ARI. Thus, ARI must be due to an impaired coupling between the egg jelly receptor and the different ion transport systems involved in AR.

4. Discussion

When sea urchin sperm are exposed to the egg jelly component that triggers the AR under ionic conditions unfavorable for this reaction, they inactivate. This is observed when normal ionic conditions are restored, but egg jelly can no longer induce AR [4,10,11]. Here, we studied the role of Ca^{2+} and Ca^{2+} channels activated by the egg jelly in inactivating sea urchin sperm. Although both ARI and AR

require the activation of the egg jelly receptor, the mechanisms that trigger these two processes are different. ARI can only be triggered by the natural inducers of this reaction, which is not the case for the AR itself [10,11]. Even though both processes require an increase in $[Ca^{2+}]_i$, ARI is much more sensitive than the AR. Reducing $[Ca^{2+}]_e$ to 2 mM is enough to inhibit AR regardless of the inducer; however, ARI is evident at this concentration.

Elevating $[Ca^{2+}]_e$ accelerates inactivation of sea urchin sperm most likely by increasing $[Ca^{2+}]_i$. However, an artificial increase in $[Ca^{2+}]_i$ is not enough to inactivate sperm, it is required that this increase occurs in the presence of the egg jelly or the egg factor. A very important finding is the inhibitory role of Mn^{2+} on ARI. This divalent cation is a well known blocker of different Ca^{2+} channels [15]. In the presence of only contaminant Ca^{2+} (0CaASW), Mn^{2+} will behave as a good inhibitor of Ca^{2+} influx. This implies that ARI requires Ca^{2+} influx to be expressed. It is also important to consider that other divalent cations can substitute for Ca^{2+} in triggering ARI, which is not the case for the AR. Sr^{2+} was almost as potent as Ca^{2+} in triggering ARI. However, the egg factor can not induce the AR, neither its associated change in pH_i, in sperm suspended in ASW0Ca with 10 mM Sr^{2+}.

We have shown here that an increase in pH_i, necessary for AR, is not required to inactivate sperm. This is in agreement with results obtained with antibodies raised against the putative egg jelly receptor. Some of them do not change pH_i but transiently increase $[Ca^{2+}]_i$ inhibiting the egg jelly-induced AR [3]. This implies that there is a domain or a subunit in the egg jelly receptor involved in triggering ARI that can be activated independently of the AR. Since ARI is an irreversible state for sperm [10], the strict requirement for egg jelly or the egg factor to induce this inactivation process indicates that it involves an irreversible modification of the egg jelly receptor [10,11]. As expected, this did not affect the ability of non-natural inducers to trigger the AR. We reported here that high pH_e could not inactivate sperm, and that previously inactivated sperm displayed normal levels of AR in response to high pH_e which activated the same Ca^{2+} channels as the egg jelly, indicating that the alteration is upstream to the activation of Ca^{2+} channels. The latter is one of the earliest responses to the egg jelly and since antibodies against the putative egg jelly receptor inhibit AR by transiently increasing $[Ca^{2+}]_i$ [3], then an altered receptor unable to activate Ca^{2+} channels should be one of the causes of ARI. A rise in pH_i of approximately 0.2 pH units is one of the requisites for AR. This rise depends on Ca^{2+} influx, as shown here (Fig. 1). However, the regulation of pH_i in sea urchin sperm seems to depend not only on Ca^{2+} since the ionomycin-induced increase in pH_i that accompanies $[Ca^{2+}]_i$ elevation is slowed down by ARI. This is further evidence to support our previously published model [5], where the egg jelly receptor signal is branched into two different, but interconnected pathways to trigger the acrosome reaction.

It is interesting that high external K^+ (30 mM) or nisoldipine (10 μM), which block AR completely when triggered by the natural inducers of the AR, are not able to inhibit the high pH_e induced AR. High external K^+ inhibits the increase in pH_i required to trigger AR, as we have shown elsewhere [5,19]. This inhibition is bypassed by high pH_e which artificially increases pH_i to the level reached during AR, and opens Ca^{2+} channels. Why does pH_e activate the two Ca^{2+} channels that participate in the AR (Fig. 6)? It has been shown that different voltage dependent or independent Ca^{2+} channels are modulated by pH. For instance, L-type Ca^{2+} channels are affected by changes in pH ([20–22]), and non-selective cation channels like the ryanodine receptor, change from being closed to fully open with a small pH increase of $\sim$0.6 pH units [23,24]. We have previously shown that the second Ca^{2+} channel involved in the AR needs an increase in pH_i to open, and shares some characteristics with the ryanodine receptor [5,25]. It is likely that the sudden change in pH_e felt by the extracellular domain of these channels may increase the open probability of one or both of them, leading to the fast but small increase in $[Ca^{2+}]_i$. Thereafter, as shown in Fig. 5(C), high pH_e causes an intracellular alcalinization which further increases Ca^{2+} uptake probably through the second channel. This could explain why nisoldipine, which just blocks the first channel, will only delay the second phase of $[Ca^{2+}]_i$ increase without inhibiting the AR when triggered by high pH_e, and also how external pH_e triggered AR without activating the egg jelly receptor.

ARI has also been documented in starfish spermatozoa [11]. When sperm from this species are treated with homologous egg-jelly in 0CaASW, they loose within a minute their ability to respond to a Ca^{2+} addition that restores its normal concentration. However, inactivated sperm undergo the AR if both, $[Ca^{2+}]_i$ and pH_i are artificially increased with ionophores [11]. The authors of this work have suggested that inactivation is due to an irreversible change(s) in the signal transduction from the receptors to the ion channels and exchangers involved in the AR [26]. Thus, the available evidence points to an irreversible modification of the egg jelly receptor as the underlying cause of ARI. In case inactivation of the acrosome reaction is also present in mammalian species, ARI could become a target for controlling the reactivity of sperm. Interestingly, it has recently been reported that the inability to attain the AR of otherwise normal human sperm could be responsible for infertility [27]. This sperm malfunction could be linked to an inactivated receptor in human sperm, an intriguing proposition that needs to be tested.

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